

Morphological Effects of Nitrogen Dioxide on the Rat Lung

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Morphological studies of the rat lung exposed to 20 ppm NO₂ for 20 hr (experiment 1), to 0.5 ppm for 19 months (experiment 2), and to 10 ppm for 14 days (experiment 3) were conducted. Changes in the mast cells of the tracheas and main bronchi of rats exposed to 0.5 ppm (experiment 4) were also observed.

In the alveolus, cytoplasmic blebbing occurred in a small number of type I cells immediately after exposure to 20 ppm NO₂ for 20 hr, and remarkable vacuolar change was observed 3 days after 10 ppm exposure. Exposure to 0.5 ppm did not cause degeneration.

Swelling and hyperplasia of type II cells were observed. The cells gradually became flat and began a transition from type II to type I cells. These intermediate-type cells were noticed in experiments 1 and 2, but no intermediate-type cells were found in experiment 3.

In each experiment, pinocytotic vesicles of endothelial cells in capillaries, followed by interstitial edema in the alveolar walls, were observed. In addition to these changes, desquamation of endothelium and widening of the endothelial junction of endothelial cells occurred in experiment 3.

The early changes observed in the animals exposed to 0.5 ppm NO₂ were the numerical and histochemical changes of mast cells in the trachea and main bronchus.

Introduction

The effects of nitrogen dioxide (NO₂) on organisms have been reported. Our study focused on detailed morphological changes in the lung caused by NO₂. Rats were exposed to NO₂ at various concentrations and for various durations and were then observed histologically and by electron microscopy.

Materials and Methods

Wistar rats were divided into the following four experimental groups.

Experiment 1: Short-term experiment to a high concentration of NO₂. Eighty-five male rats weighing 100 to 150 g were exposed to 20 ppm NO₂ for 20 hrs. After the exposure, the rats were maintained in clean air and killed at 24, 36, 48, or 60 hrs or days 3, 5, 7, 10, 15, 20, 25, 30, or 35.

Experiment 2: Long-term exposure experiment to a low concentration of NO₂. Eighty-six rats weighing 100 to 300 g were continuously exposed to 0.5 ppm NO₂ and killed at 7 or 15 days, or at 1, 2, 4, 6, 12, or 19 months.

Experiment 3: Medium-term exposure experiment with a high concentration of NO₂. Eighteen rats

weighing 100g were continuously exposed to 10 ppm NO₂ and killed at 3, 7, or 14 days.

Experiment 4: Exposure experiment to a low concentration of NO₂ for changes in mast cells in the wall of the trachea and main bronchus. One hundred sixty rats weighing 80 to 170 g were continuously exposed to 0.5 ppm NO₂ and killed at 5, 10, 20, or 45 min; at 1, 2, 3, 4, 6, 8, 10, or 24 hrs, or at 2, 4, or 6 days.

Rats in experimental groups were compared with their corresponding control (unexposed) groups.

In experiments 1, 2, and 3, one lung and the trachea from each rat were removed, and 3% glutaraldehyde in cacodylate buffer was perfused through the trachea. The lung was postfixed in 1% osmium tetroxide, embedded in Epon, and examined by electron microscopy (HITACHI HU-12). For light microscopy, 1- μ m sections were cut from each block, mounted on glass slides, and stained with toluidine blue. The remaining lungs were fixed in 10% formalin, and paraffin-embedded sections were also examined.

In experiment 1, in order to estimate the change in number of the alveolar epithelial cells, the number of type I and type II cells were calculated by using the number of these cells observed in 30 meshholes of the DN150 mesh.

In experiment 4, the tracheas and the bronchi were fixed in lead subacetate-formalin solution, and the paraffin-embedded sections were stained with toluidine blue, alcian blue-safranin (AB-S).

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FIGURE 1. Lung section from a rat exposed to 20 ppm NO_2 for 20 hr and killed immediately after exposure showing blebbing in alveolar type I cell. $\times 8000$.

Some of the tracheas of rats exposed to 0.5 ppm NO_2 were fixed with Carnoy's solution and treated by the vacuum freeze-drying and paraffin embedding method. For the observation of peritoneal mast cells, thick drops of peritoneal fluid were transferred to slides and fixed in paraformaldehyde vapor. Slides were examined with a fluorescence microscope according to the *o*-phthalaldehyde fluorescent method (1) for histamine fluorescence. Furthermore, homogenized tissues were examined for quantitative analysis of histamine of tracheas according to the Shore's method (2).

Results

Experiment 1

When Epon-embedded 1- μm thick sections stained with toluidine blue were examined by light microscopy, the nuclear contour of type I and type II alveolar cells and the cytoplasm of type II cells were clearly identified. These cells were found evenly on the alveolar wall in the lungs of control rats. While no differences between the experimental and the control groups were discovered within 3 days after the exposure, notable swelling of type II alveolar cells and thickening of the alveolar walls in the experimental group were observed between 5 and 15 days after the exposure. However, these changes were not clearly recognized in the paraffin-embedded sections.

On observing the rats in the control group under electron microscopy, the inner surface of the alveolar walls were covered with flat type I and cuboidal type II alveolar cells. The type II cells protruded more than the type I cells and had multilamellar bodies within the cytoplasm and apical microvilli. In the interstitium of the alveolar walls, collagen fibers were regularly arranged, and in the endothelial cells of capillaries, pinocytotic vesicles of almost the same size were regularly distributed.

In the experimental groups, the rats examined between 24 and 48 hr after the exposure showed partial cytoplasmic blebbing in a small number of type I alveolar cells (Fig. 1). Swelling and hyperplasia of type II alveolar cells were observed in the 3 days after the exposure. These changes in type II cells became more remarkable, and multilamellar bodies were increased in number 5 to 15 days after exposure (Fig. 2). Twenty days after exposure, swelling of type II alveolar cells was less conspicuous, and microvilli and multilamellar bodies decreased.

Twenty-five days after exposure, type II cells became flatter, similar to type I alveolar cells. However, the remnants of multilamellar bodies and some microvilli were observed, so these flatter type II cells can be called intermediate-type cells (Figs. 3 and 4). A small number of intermediate cells were first observed in the 15-day group, and the number of cells increased in the 20- and 25-day groups and decreased in the 30-day group. In

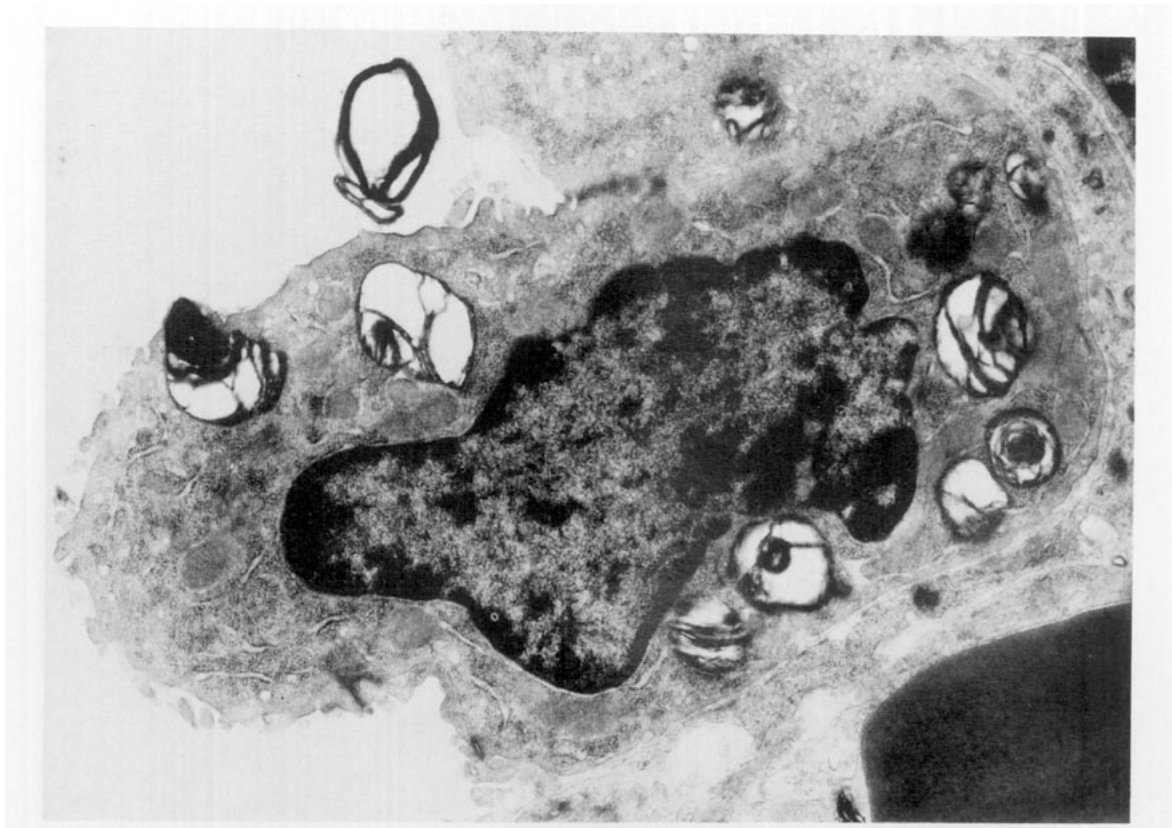


FIGURE 2. Lung section from a rat exposed to 20 ppm NO₂ for 20 hr and killed 5 days after last exposure showing a number of lamellar bodies in enlarged alveolar type II cells. $\times 13,000$.

Table 1. Ratio of type I and type II alveolar cells in the alveolus of the lung of a rat exposed to 20 ppm NO₂ for 20 hr and then maintained in the clean air.

Time after exposure	Type I cells, %	Type II cells, %
Hr		
0	26.8	73.2
36	26.7	73.3
48	27.3	72.7
60	29.3	70.7
Days		
3	24.0	76.0
5	25.1	74.9
10	20.6	79.4
15	16.3	83.7 (5.0)
20	37.3 (4.3)*	62.7 (8.1)
25	23.8 (8.2)	76.2 (7.0)
30	29.1 (6.9)	70.9 (1.3)
35	29.7	70.3
Control	30.2	69.8

* Numbers in parentheses indicate percent intermediate type cells.

the 35-day group, intermediate-type cells were not observed, and the lungs showed the same histology as in the control group.

Table 1 shows the number of type I and type II alveolar cells of the alveolus. The ratio for type II alveolar cells increased dramatically in the 10-day and 15-day groups, and as mentioned previously, the intermediate-type cells increased in the groups sacrificed at 20 days or more.

In the alveolar interstitium, the changes of capillary endothelial cells were observed initially. The pinocytotic vesicles increased in size and number in the 3-day group, and such changes were also observed in the 20-day group. Moreover, interstitial edema was observed in the 5-day group (Fig. 5), was more severe in the 10-day group, and became less conspicuous in the 20- and 25-day groups. No widening of the junctions of the endothelial cells of capillaries was observed.

Experiment 2

No conspicuous histological changes were observed by means of the 1- μ m thick section method in the lungs of rats exposed long term to 0.5 ppm NO₂ in the 2-month group. In the 4-month group and in groups exposed more than 4 months, however, there was swelling of type II alveolar cells. In the 6-month group, the width of the alveolar wall increased. In the 19-month group, slight fibrous thickening of the pleura was found with the use of Azan-Mallory staining, but there was no fibrosis in the lung parenchyma.

The same changes in experiment 1 were observed by electron microscopy later in this experiment. Swelling of type II alveolar cells and an increased number of lamellar bodies were found in the 2-month group, and these changes became more conspicuous. The increase of lamellar bodies and the secretion of the contents were

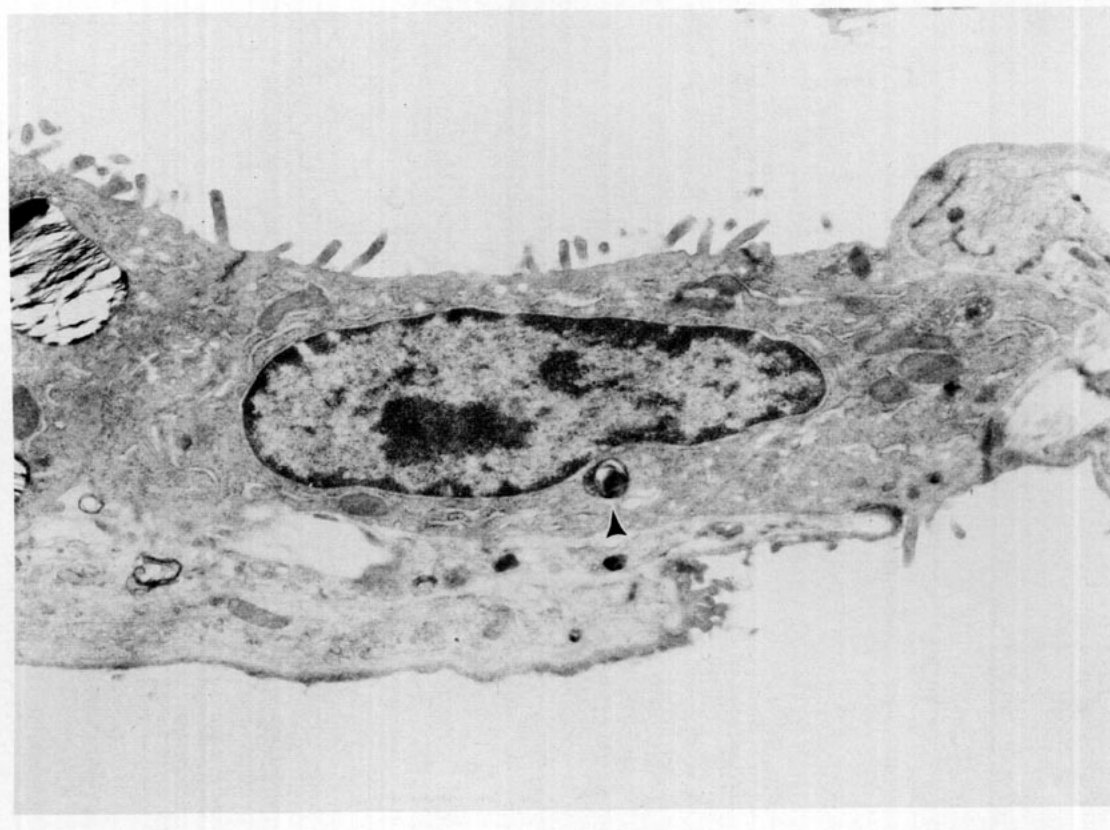


FIGURE 3. A flattened type II cell (intermediate-type cell) with lamellar bodies (▲) in a rat exposed for 20 hrs to 20 ppm NO_2 and killed 20 days after last exposure. $\times 11,000$.

also recognized (Fig. 6). These changes were also observed in the 19-month group.

A small number of intermediate-type alveolar cells observed in experiment 1 were also found in each group after 4 months in this experiment. On the other hand, in the alveolar wall of the 2-month group, pinocytotic vesicles in the capillary endothelial cells became more prominent. In the 4-month group, interstitial edema was present. Interstitial edema increased in degree in groups of 6 months or more. Alveolar interstitium showed marked edema in the 12-month group (Fig. 7); these changes were also found in the 19-month group. As in experiment 1, no widening of junctions of capillary endothelial cells was observed.

Experiment 3

A histological observation on 1- μm thick sections of the lungs of the rats exposed to 10 ppm NO_2 showed swelling of type II alveolar cells in the 3-day group, desquamation of alveolar cells, slight thickening of the alveolar wall, and swelling of nonciliated bronchiolar cells in the 7- and 14-day groups. This experiment showed some changes in the bronchi and tracheas, an increase of goblet cells in the 3-day group, slight infiltration of inflammatory cells and falling-off of cilia in the 7-day group, and partial desquamation of the bronchial epithelium in the 14-day group.

Type I alveolar cells exhibited irregular swelling on

the surface of the cells and vacuolar degeneration of the cytoplasm in the 3-day group, as seen with electron microscopy (Fig. 8). Swelling and numerical increase of type II alveolar cells were accompanied by an increase of multilamellar bodies in number and in size. Extrusion of the multilamellar bodies and vacuolar formation in the cytoplasm were obvious (Fig. 9). The alteration of type I alveolar cells became obvious, the cells desquamated from the basement membrane, the vacuolar degeneration of type II alveolar cells became conspicuous, and pycnosis of the nuclei was also observed in the 7- and 14-day groups. The transformation from type II to type I cells observed in experiment 1 and 2 was not observed here.

In the alveolar wall, the degeneration of the endothelial cells of capillaries was obvious; in the 3-day group, pinocytotic vesicles not only increased in number and size but also formed vacuoles, some of which became cystic (Fig. 10). These changes became more pronounced as exposure time increased. The endothelial cells desquamated from the basement membrane, and the junctions of the endothelial cells widened where transudation of erythrocytes was observed in the 14-day group. Slight interstitial edema was found in the 3-day group and was obvious in the 7- and 14-day groups.

In the bronchioles, nonciliated cells showed cytoplasmic rarefaction in the 7-day group and marked vacuolar degeneration in the 14-day group.

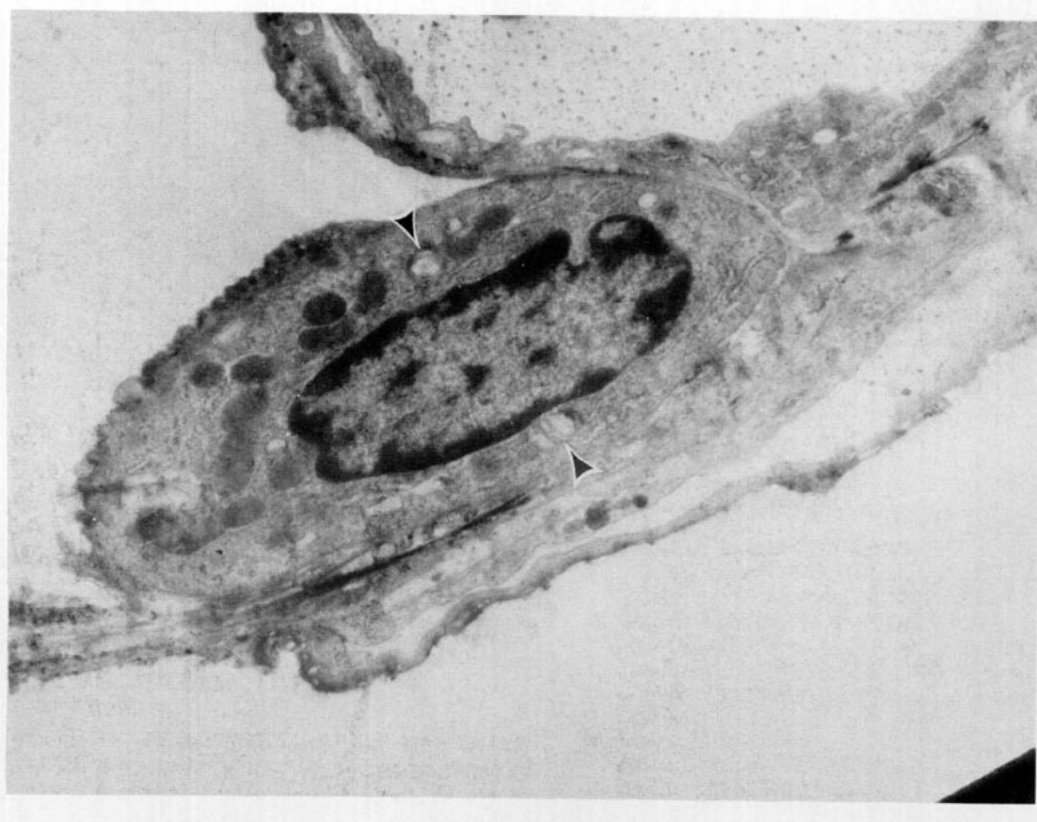


FIGURE 4. An intermediate-type cell resembling an alveolar type I cell in a rat exposed for 20 hr to 20 ppm NO₂ and killed 25 days after last exposure. Note the traces of lamellar bodies (▲). $\times 11,000$.

Table 2. Histamine content in tracheas of rats exposed to 0.5 ppm NO₂.

NO ₂ exposure duration	Histamine, γ/g tissue		<i>n</i> ^a	Significance, <i>p</i> value
	Mean \pm SD			
Min				
0	23.9 \pm 10.1	11		
10	20.2 \pm 8.2	7		> 0.4
30	14.6 \pm 6.5	6		> 0.05
45	10.1 \pm 4.6	8		< 0.01
Hr				
1	10.0 \pm 3.4	3		< 0.05
4	18.0 \pm 5.4	8		> 0.1
6	20.1 \pm 12.4	4		> 0.5
10	16.6 \pm 1.7	2		> 0.3
Days				
1	19.7 \pm 3.7	2		> 0.6
3	16.9 \pm 4.8	4		> 0.2
5	18.0 \pm 4.9	4		> 0.3
7	17.0 \pm 2.2	4		> 0.3

^a Rats weighed 70–130 g.

Experiment 4

In the group exposed to 0.5 ppm NO₂, mast cells were abundant on the trachea and main bronchus (Figs. 11 and 12). On the tracheal wall, the number of mast cells per millimeter length increased as the duration of exposure increased: 13.2/mm for the control group, 24.8/

mm after 30-min exposure, and 32.9/mm after 96-hr exposure. Degranulation of mast cells was also observed in the samples after 4-hr exposure.

The control groups had two types of cells classified by the reaction to the AB-S staining: one type of tracheal and bronchial mast cells partially stained by both alcian blue and safranin, and one type colored in most parts of cells only by alcian blue or only by safranin. Stainability in mast cells changed markedly after NO₂ exposure. After 30-min exposure, almost all the cells were stained only by alcian blue; this phenomenon was also observed after 10 hr exposure. After 24-hr exposure, stainability of mast cells was the same as in control groups.

The *o*-phthalaldehyde fluorescence method was employed to examine mast cells in the tracheas and peritoneal fluid for the study of changes in stainability and the significance of these changes. As observed with histamine fluorescence, mast cells that reacted to safranin dense-positively emitted strong fluorescent light that became weaker as the cells reacted positively to alcian blue. Additionally, Shore's method was employed to quantify the histamine in tracheal tissues. After 45- and 60-min exposures, the histamine content decreased significantly ($p < 0.01$, $p < 0.05$) (Table 2). This decrease corresponded to the time when the cells began to turn blue in AB-S-stained specimens.

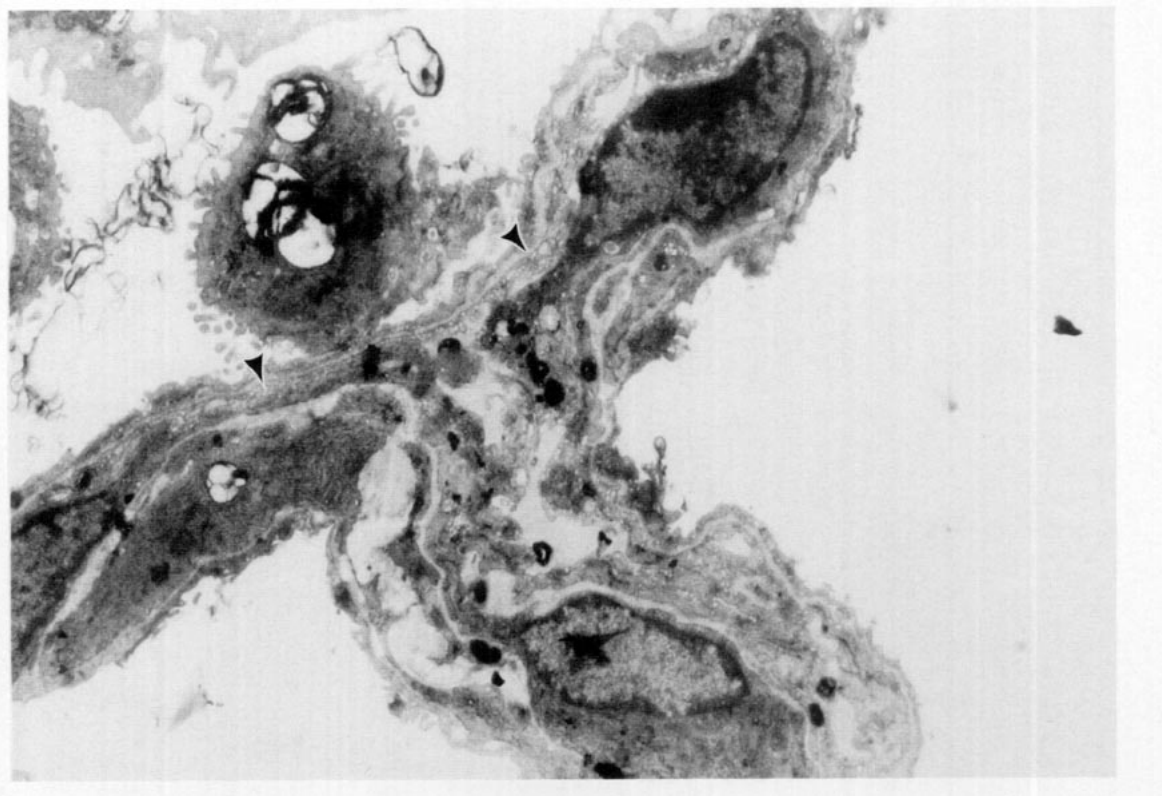


FIGURE 5. Pinocytotic vesicles in capillary endothelial cells (▲) in a rat exposed for 20 hr to 20 ppm NO_2 and killed 5 days after last exposure. $\times 5000$.

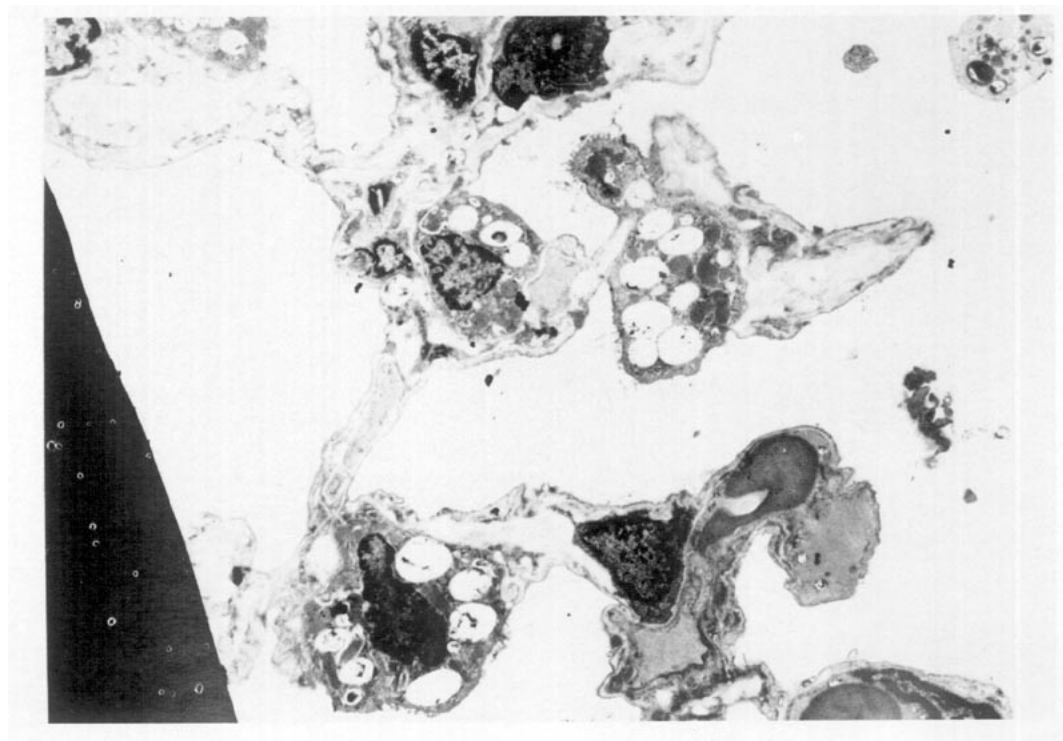


FIGURE 6. Increased alveolar type II cells in a rat after 4 months of continuous exposure to 0.5 ppm NO_2 . $\times 2000$.



FIGURE 7. Marked edema of interstitial connective tissue in a rat after 12 months of continuous exposure to 0.5 ppm NO₂. $\times 15,000$.

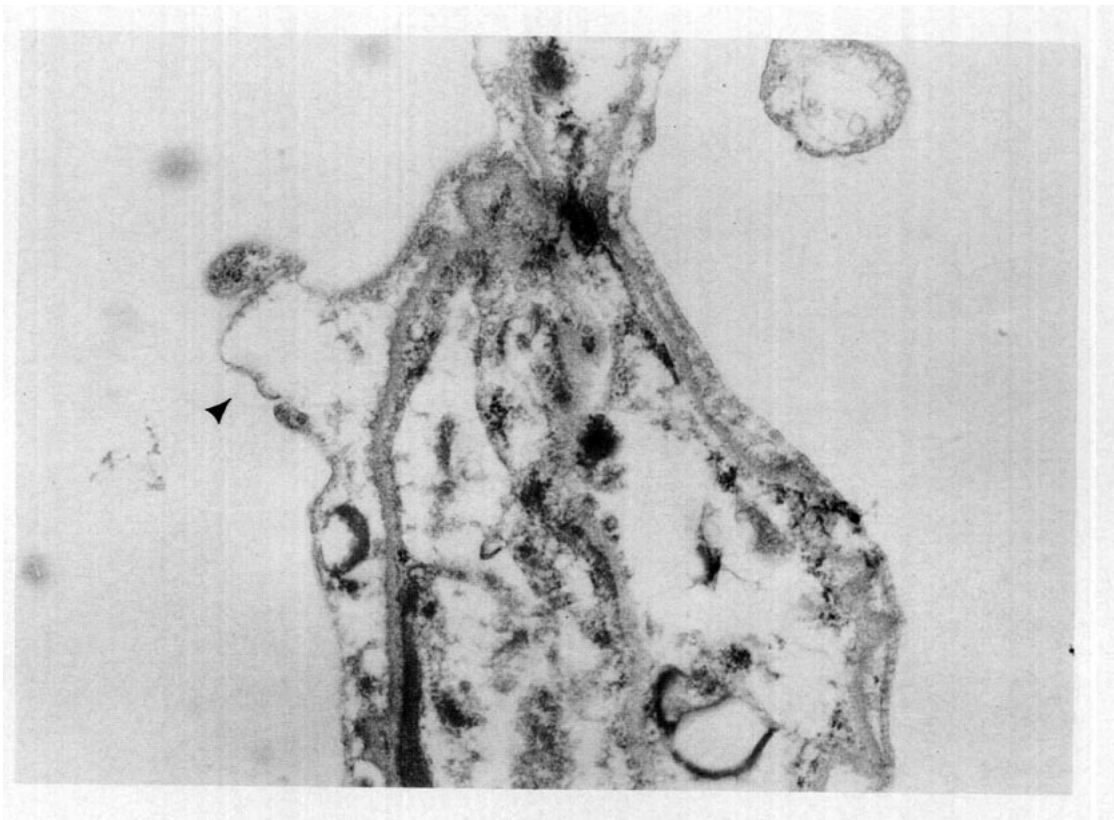


FIGURE 8. Vacuolar degeneration of type I alveolar cells in a rat after 3 days of continuous exposure to 10 ppm NO₂. $\times 11,000$.

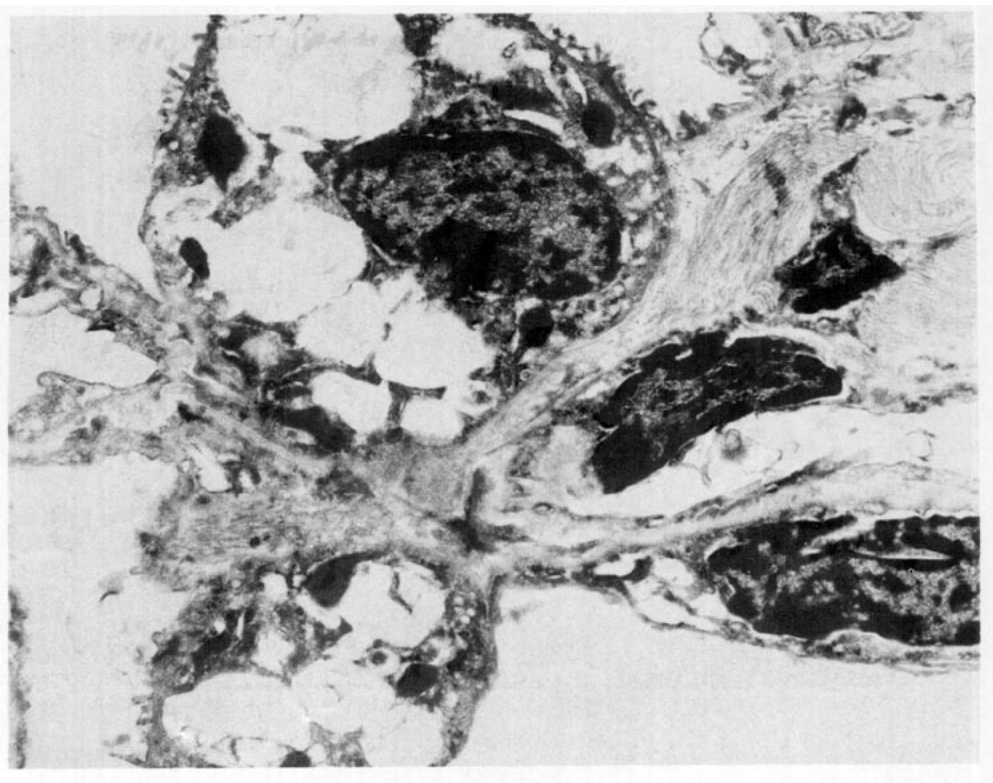


FIGURE 9. Vacuolar degeneration of type II alveolar cells in a rat after 7 days of continuous exposure to 10 ppm NO_2 . $\times 6000$.

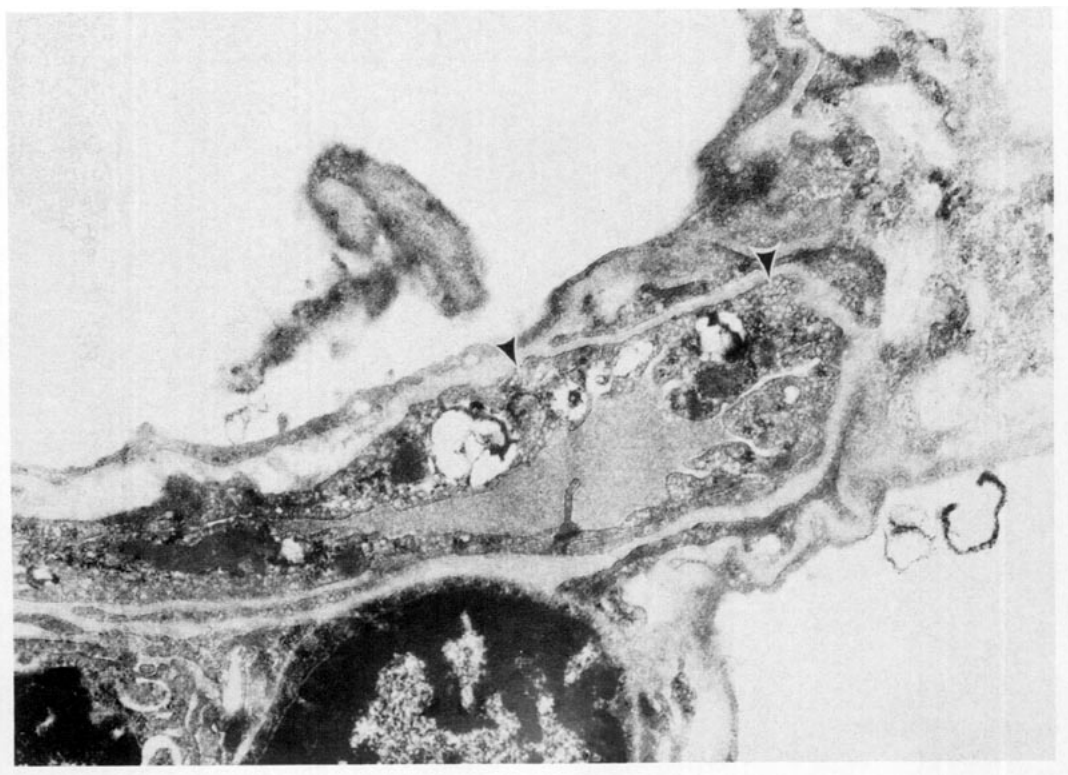


FIGURE 10. Pinocytotic vesicles (\blacktriangle) in capillary endothelial cells with vacuolar degeneration in a rat after 14 days of continuous exposure to 10 ppm NO_2 . $\times 7000$.

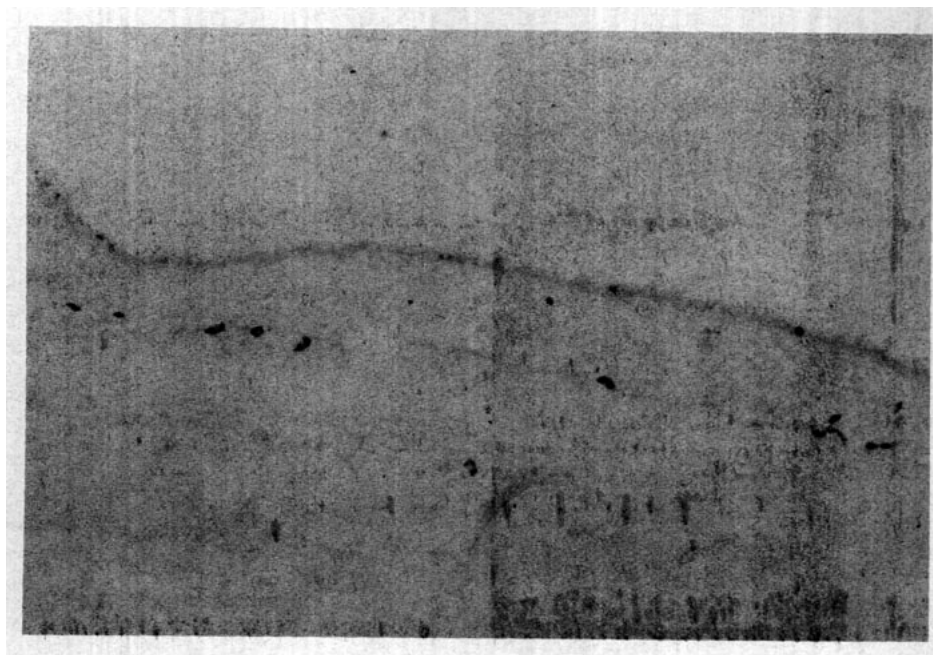


FIGURE 11. Tracheal wall of normal control. A small number of mast cells are scattered. Toluidine blue stain. $\times 100$.

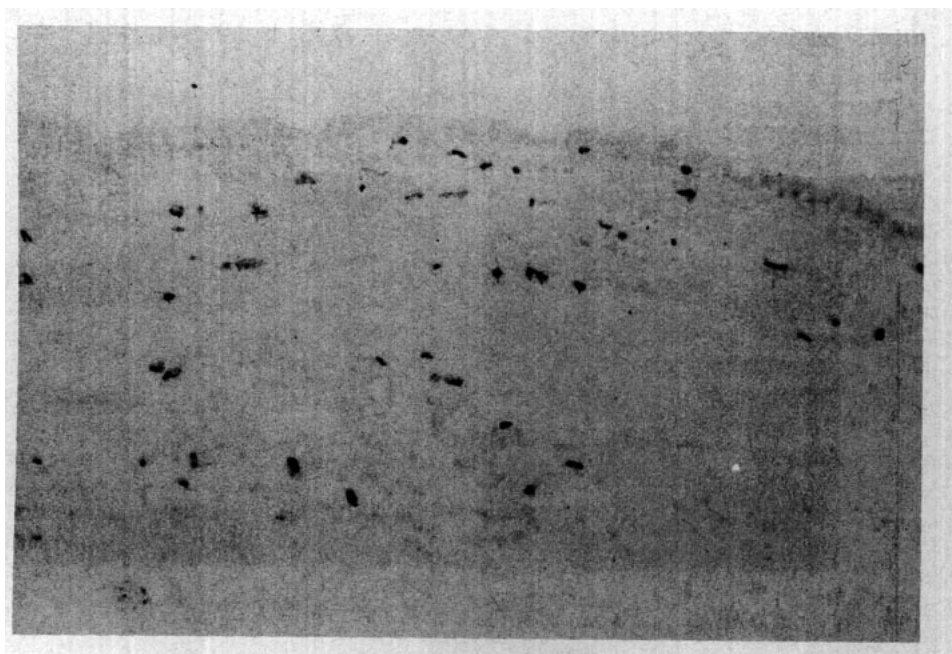


FIGURE 12. Tracheal wall of a rat after 24 hr exposure to 0.5 ppm NO₂. Mast cells are numerous in the submucosa. Toluidine blue stain. $\times 100$.

Discussion

The changes in the lungs of rats exposed to NO₂ at various levels were observed in the alveolar epithelial cells and the capillaries of the alveolar walls (experiments 1, 2, and 3). In the experimental group exposed to a high concentration (20 ppm) for 20 hr, slight degeneration was observed in a small number of type I alveolar epithelial cells after the discontinuation of exposure; swelling and hyperplasia of type II alveolar ep-

ithelial cells followed, and the reparation of the epithelium terminated with the transformation of type II cells to type I. The morphological and numerical changes in both types of cells are consistent with the reports of Evans et al. (3-9) and Sherwin et al. (10,11). The fact that type II cells transform to type I has confirmed the results Evans et al. (4) found with the use of autoradiography.

As the swelling of type II cells occurred, capillary permeability accelerated, and edema of the alveolar

walls was observed. The results of our experiments support the increase of protein in the dialyzed pulmonary lavage fluid and the rise of plasma levels in tritiated serum that Sherwin et al. (12,13) reported. Such changes influence the exchange of gases between blood and air. It is reported that interstitial edema was observed after 30 to 40 days continuous exposure to 0.5 ppm NO₂ (14).

The changes caused by the long-term exposure to 0.5 ppm NO₂ are morphologically similar to those caused by the short-term exposure to 20 ppm NO₂, although the time of manifestation of the changes is different. The change in the epithelium observed 3 days after 20 hr exposure to 20 ppm NO₂ is similar to that of 2 months of continuous exposure to 0.5 ppm NO₂. The interstitial edema observed 5 days after the 20 ppm exposure was also observed 4 months after the 0.5 ppm NO₂ exposure. These results show that the effect of NO₂ can be evaluated by multiplying the level of NO₂ by the exposure duration within certain limits of exposure time and NO₂ density. According to Evans et al. (8), in the long-term exposure experiments to 2 ppm NO₂, the labeling index of type II alveolar epithelial cells of rats increases only 2 or 3 days after the exposure. Freeman et al. (15) reported that if the rats survived to their natural life-spans after 2 ppm NO₂ exposure, hypertrophy of the alveolar lining epithelium was marked.

In addition to the slight changes of type I alveolar cells in experiment 1, the swelling of type II alveolar cells and numerical increase of pinocytotic vesicles of the capillary endothelial cells can be considered as changes which indicate the accelerated responsive functions of these cells. These changes are obvious in experiments 1 and 2. In the 19-month group exposed to a low concentration of NO₂, there was slight fibrous thickening of the pleura. Freeman et al. (16) also reported that collagen and elastic fibers increased in the lungs of rats exposed to 15 ppm NO₂ for 20 weeks.

In experiment 3, distinct degeneration was observed: desquamation and vacuolar degeneration of type I alveolar cells and endothelial cells; pycnosis of the nuclei of type II alveolar cells; and the disconnection of the junctions of endothelial cells. The level of NO₂ used in experiment 3 is half of that in experiment 1, while the exposure duration is three times longer than that of experiment 1. This suggests that the exposure duration has a significant role. According to Dwell et al. (17), the degeneration of type I alveolar cells (blebbing of cytoplasm, degeneration of mitochondria) and swelling of the endothelial cells were always observed in beagle dogs exposed to 3 to 12 ppm NO₂. The changes observed in our experiment were less severe than those in Dwell's experiment. According to Azoulay-Dupuis et al. (18), rats were more tolerant than guinea pigs to NO₂, but newborns of both species were less affected than adults.

As seen in our studies of the trachea and bronchus, researchers consider the histochemical changes of the mast cells observed 30 min after exposure to 0.5 ppm NO₂ to be the earliest morphological changes. Thomas

et al. (19) reported that the degranulation of mast cells of the lungs of 0.5 ppm NO₂-exposed rats was observed 4 hr after the exposure. The histamine effect should be investigated further.

Conclusion

The purpose of this study was to observe the morphological changes in the lungs of male Wistar rats exposed to various levels of concentration of NO₂ for various periods of time. Animals were exposed to 20 ppm for 20 hr, then maintained in the clean air for 24 hr to 35 days (experiment 1). Rats were also exposed continuously to 0.5 ppm up to 19 months (experiment 2), and another group was exposed to 10 ppm for 14 days (experiment 3). These animals were sacrificed at various periods, and were then observed histologically and by electron microscopy. In order to investigate the changes of mast cells in the trachea and bronchus, rats were exposed to 0.5 ppm NO₂ for 6 days and studied histologically.

The changes caused by NO₂ were found in the alveolar epithelium and the alveolar interstitium. In experiment 1, cytoplasmic blebbing was observed in a small number of type I alveolar epithelial cells, while in experiment 3, where rats were continuously exposed to a level of NO₂ 50% lower in concentration than in experiment 1, considerable changes were observed in type I cells. Degeneration of type I cells was not found in exposure to the low concentration of NO₂ in experiment 2.

Cytoplasmic blebbing was followed by the swelling and hyperplasia of type II alveolar cells, after which the cells gradually become flat and began a transition from type II to type I cells. These intermediate types of alveolar epithelial cells were observed in experiments 1 and 2, but no such type of cells was found in experiment 3.

Pinocytotic vesicles of endothelial cells of capillaries, followed by interstitial edema in the alveolar walls, were observed in experiments 1 and 2. In addition to these changes, desquamation and widening of the junctions of endothelial cells were observed in experiment 3.

The changes observed at early stages in animals exposed to 0.5 ppm NO₂ were the numerical increase and histochemical changes of mast cells in the trachea and bronchus.

REFERENCES

1. Enerbäck, L. Detection of histamine in mast cell by o-phthalaldehyde reaction after liquid fixation. *J. Histochem. Cytochem.* 17: 757-759 (1969).
2. Shore, P. A., Burkhalter, A., and Cohn, V. H. A method for the fluorometric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.* 127: 182-186 (1959).
3. Evans, M. J., Stephens, R. J., and Freeman, G. Effects of nitrogen dioxide on cell renewal in the rat lung. *Arch. Intern. Med.* 128: 57-60 (1971).
4. Evans, M. J., Cabral, L. J., Stephens, R. J., and Freeman, G.

- Renewal of alveolar epithelium in the rat following exposure to NO₂. *Am. J. Pathol.* 70: 175-198 (1973).
5. Evans, J. J., Cabral, L. J., Stephens, R. J., and Freeman, G. Acute kinetic response and renewal of the alveolar epithelium following injury by nitrogen dioxide. *Chest* 65 (suppl.): 62S-65S (1974).
 6. Evans, M. J., Cabral, L. J., Stephens, R. J., and Freeman, G. Transformation of alveolar type II cells to type I cells following exposure to NO₂. *Exp. Mol. Pathol.* 22: 142-150 (1975).
 7. Evans, M. J., and Dekker, N. P., Cabral-Anderson, L. J. Freeman, G. Quantitation of damage to the alveolar epithelium by means of type II cell proliferation. *Am. Rev. Respir. Dis.* 118: 787-790 (1978).
 8. Evans, M. J., Stephens, R. J., Cabral, L. J., and Freeman, G. Cell renewal in the lungs of rats exposed to low levels of NO₂. *Arch. Environ. Health* 24: 180-188 (1972).
 9. Cabral-Anderson, L. J., Evans, M. J., and Freeman, G. Effects of NO₂ on the lungs of aging rats. *Exp. Mol. Pathol.* 27: 353-365 (1977).
 10. Yuen, T. G. H., and Sherwin, R. P. Hyperplasia of type II pneumocytes and nitrogen dioxide (10 ppm) exposure. *Arch. Environ. Health* 22: 178-188 (1971).
 11. Sherwin, R. P., Dibble, J., and Weiner, J. Alveolar wall cells of the guinea pig. *Arch. Environ. Health* 24: 43-47 (1972).
 12. Sherwin, R. P., Richers, V. Lung capillary permeability. *Arch. Intern. Med.* 128: 61-68 (1971).
 13. Sherwin, R. P., and Carlson, D. A. protein content of lung lavage fluid of guinea pigs exposed to 0.4 ppm nitrogen dioxide. *Arch. Environ. Health* 27: 90-93 (1973).
 14. Hattori, S., and Takemura, K. Ultrastructural changes in the bronchial alveolar system caused by air pollution and smoking. *J. Clin. Electron Microsc.* 6: 350 (1974).
 15. Freeman, G., Crane, S. C., Stephens, R. J., and Furiosi, N. J. Environmental factors in emphysema and a model system with NO. *Yale J. Biol. Med.* 40: 566-575 (1968).
 16. Freeman, G., Crane, S. C., and Furiosi, N. J. Healing in rat lung after subacute exposure to nitrogen dioxide. *Am. Rev. Respir. Dis.* 100: 662-676 (1969).
 17. Dowell, A. R., Kilburn, K. H., and Pratt, P. C. Short term exposure to nitrogen dioxide. Effects on pulmonary ultrastructure, compliance and the surfactant system. *Arch. Intern. Med.* 128: 74-80 (1971).
 18. Azoulay-Dupuis, E., Torres, M., Soler, P., and Moreau, J. Pulmonary NO₂ toxicity in neonate and adult guinea pigs and rats. *Environ. Res.* 30: 322-339 (1983).
 19. Thomas, H. V., Mueller, P. K., and Wright, R. Response of rat lung mast cells to nitrogen dioxide inhalation. *J. Air Pollut. Control Assoc.* 17: 33-35 (1967).